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Differential Translocation of Nuclear Factor-KappaB in a Cardiac Muscle Cell Line Under Gravitational Changes

Ohwon Kwon,
Department of Mechanical Engineering, University of Cincinnati, Cincinnati, OH 45221

Michael Tranter,
Department of Pharmacology and Cell Biophysics, University of Cincinnati, Cincinnati, OH 45221

W. Keith Jones,
Department of Pharmacology and Cell Biophysics, University of Cincinnati, Cincinnati, OH 45221

John M. Sankovic, and
Microgravity Science Division, NASA Glenn Research Center, Cleveland, OH 44135

Rupak K. Banerjee[1]
Department of Mechanical Engineering, and Department of Biomedical Engineering, University of Cincinnati, Cincinnati, OH 45221

Abstract

Microgravity (micro-g) environments have been shown to elicit dysregulation of specific genes in a wide assay of cell types. It is known that the activation of transcription factors and molecular signaling pathways influence various physiological outcomes associated with stress and adaptive responses. Nuclear factor-kappa B (NF-κB) is one of the most prevailing oxidation-sensitive transcription factors. It is hypothesized that simulated microgravity would activate NF-κB and its downstream transcriptional networks, thus suggesting a role for NF-κB in microgravity induced muscle atrophy. To investigate the activation of NF-κB in a rat cardiac cell line (H9c2) under micro-g, rotating wall vessel bioreactors were used to simulate micro-g conditions. Western blotting revealed that mean nuclear translocation of NF-κB p65 subunit was 69% for micro-g and 46% for unit-g dynamic control as compared with a 30 min TNF-α positive control ($p < 0.05$, $n = 3$). The results from western blots were confirmed by enzymelinked immunosorbent assay, which showed 66% for micro-g and 45% for dynamic control as compared with positive control ($p < 0.05$, $n = 3$). These results show significant differential translocation of NF-κB p65 under simulated micro-g. These results may be expanded upon to explain physiological changes such as muscle atrophy and further identify the regulatory pathways and effector molecules activated under exposure to micro-g.

1 Introduction

It has been demonstrated that there are basic physiological changes including reduced metabolism, immune response, anemia, and loss of bone and muscle mass under microgravity (micro-g, MG) [1,2]. Rotating wall vessel (RWV) bioreactors developed by NASA have been used in the laboratory to create suspension cell culture environments for simulating micro-g conditions on earth [3]. These bioreactors have been used to investigate

[1]Corresponding author. rupak.banerjee@uc.edu.

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cellular responses to the change in gravitational forces [4,5]. For instance, in our preliminary results from DNA microarrays with rat PC12 cells, it was shown that over 100 genes were dysregulated more than twofold under simulated micro-g in the RWV culture. Among the regulated genes, those involved in the oxidoreductase activity category were most significantly differentially expressed [6]. Although the microarray technique dealing with mRNA expression can identify differences in the expression level of thousands of gene products [7], it has a higher degree of variability that requires validation of the data by secondary techniques such as protein expression [8]. In addition, it has been shown that there is not always a linear expression correlation between mRNA and the encoded protein [9].

It is known that the activation of transcription factors and molecular signaling pathways influence various physiological outcomes associated with stress and adaptive responses [10]. One of the most prevailing oxidation-sensitive transcription factors is the nuclear factor-kappaB (NF-κB) [11,12]. Interestingly, NF-κB activation has also been implicated in muscle atrophy [13]. NF-κB exists as a dimer of related proteins including NF-κB1 (p50), NF-κB2 (p52), Rel A (p65), Rel B, and c-Rel. NF-κB dimers exist in the cytoplasm bound to inhibitory proteins (IκBs) that prevent nuclear translocation of the transcription factor under basal conditions. Stimulation of cells by such factors as oxidative stress induces a signaling pathway that initiates ubiquitination and destruction of the IκBs, which then allows nuclear translocation of the free NF-κB dimers. The p65 subunit is the major transactivating member of the Rel family of proteins and was thus chosen as the primary protein target for this study.

For this study, a cell line derived from rat ventricular heart muscle cells (H9C2) [14] was used to investigate the effect of micro-g on the activation of NF-κB. H9c2 cells have been used as an in vitro model of both skeletal and cardiac muscles, since they can exhibit the physical and biochemical properties of both tissues [15]. It is hypothesized that NF-κB will be activated differentially under simulated micro-g conditions, and micro-g induced activation of downstream NF-κB transcriptional networks will induce a muscle atrophy response in a cardiomyocyte cell line. Therefore, the objective of the present study is to investigate the activation of NF-κB in a cardiac muscle cell line under simulated micro-g in the RWV culture and to further provide a context to identify the regulatory pathways and effector networks involved in the response to the physiological changes [16] induced by exposure to micro-g.

2 Experimental Materials and Methodology

2.1 Cell Culture

H9c2 cells were maintained in growth medium composed of Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA)) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. For culturing in the RWV, H9c2 cells (4×10^5 cells/ml) were allowed to attach on the surfaces of microcarrier beads (Cytodex-3, Amersham Biosciences, Piscataway, NJ) for 2 h in the RWV in a humidified incubator at 37°C and 5% CO_2 prior to rotation of the reactors. The RWV bioreactors were oriented to expose microcarrier beads with non-confluent adherent cells to conditions of simulated micro-g (Fig. 1(*a*)) and normal unit-g by simply changing the rotational plane of the bioreactor to serve as dynamic control (DC, Fig. 1(*b*)). T-75 flasks cultured to ~80% confluency were treated with TNF-α (25 ng/ml) for 30 min as a positive (+) control (Fig. 1(*c*)). The RWVswere rotated at an initial speed of 10 rpm, and the speed was increased as the cell/bead aggregates were enlarged [17].

2.2 Nuclear Extracts

Cell samples were homogenized at low speed in buffer A (10 mM HEPES (*p*H 9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 25 μg/ml leupeptin, 0.2 mM sodium orthovanadate, and 0.1% (v/v) Triton X), vortexed, and incubated on ice for 10 min. After centrifugation (5000 g for 10 min), the pellets were suspended in solution C (20 mM HEPES, (pH 7.9), 25% (v/v) glycerol, 0.6 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 25 μg/ml leupeptin, and 0.2 mM sodium orthovanadate). This suspension was incubated on ice for 40 min with rigorous vortexing every 10 min. After centrifugation (10,000 g for 15 min), the supernatant was retained as a crude nuclear extract. Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard.

2.3 Western Blotting

Western blots were prepared for p65 based on the protocol described previously [18]. Cell lysates were run on 12% polyacrylamide gels, and protein was transferred to 0.45 μm nitrocellulose filters (Amersham Bioscience, Piscataway, NJ). Filters were blocked in 5% nonfat dry milk in trisbuffered saline (TTBS (10 mM Tris HCl (*p*H 7.2) and 0.15 M NaCl) +0.1% Tween 20) for 1 h (RT) and incubated for 2 h (RT) with a primary NF-κB p65 antibody (sc-372, 1:10,000 dilution; Santa Cruz Biotechnology, Inc., CA) and rabbit anti-actin (A-2066, 1:50,000 dilution; Sigma, St. Louis, MO) in TTBS that contained 5% nonfat dry milk, washed three times for 5 min with TTBS, and then incubated for 2 h with a secondary goat anti-rabbit IgG-HRP antibody (sc-2054, 1:10,000 dilution; Santa Cruz. Biotechnology, Inc., CA). Blots were again washed three times for 5 min with TTBS, and signals were detected by enhanced chemiluminescence (ECL) kit (NEL 104; Perkin Elmer, Waltham, MA). The blots were exposed to Kodak BioMax Light films and densitometry was done using National Institutes of Health (NIH) image software. The signals were normalized to actin bands to account for protein loading and transfer variability.

2.4 Enzyme-Linked Immunosorbent Assay

The DNA binding of the NF-κB p65 subunit in nuclear extract was also assessed by enzyme-linked immunosorbent assay (ELISA) designed using Pierce NeutrAvidin coated 96 well plates (Cat No. 15116; Pierce, Rockford, IL). A biotinylated capture oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′) was bound to each well at a binding capacity of 15 pmoles of biotin per well for 2 h in tris-buffered saline (TBS) containing 0.05% Tween 20. Nuclear lysates (3 μg/well) were incubated in wells for 45 min, primary anti-p65 antibody (sc-372, 1:5000 dilution; Santa Cruz. Biotechnology, Inc., CA) for 30 min, and secondary HRP-conjugated antibody (sc-2054, 1:5000 dilution; Santa Cruz. Biotechnology, Inc., CA) for 30 min. Chemiluminescent reagent (NEL 104; Perkin Elmer, Waltham, MA) was added for 5 min and luminescence was detected using a Tecan plate reader. Each well was washed three times with tris-buffered saline containing 0.05% Tween 20 between each step. All incubations were performed at room temperature.

2.5 Statistical Analysis

Student's *t*-test and ANOVA were performed using Excel (Microsoft, Redmond, WA) and a *P* value <0.05 was considered to be significant. All data were expressed as mean ± SEM from at least three independent experiments.

3 Results and Discussion

To investigate the effect of micro-g conditions on the regulation of the oxidation-sensitive transcription factor, NF-κB, three different groups of cells—(1) MG, (2) DC in the RWV

bioreactor, and (3) stationary Petri dishes for the TNF-α (+) control—were cultured and prepared for the analyses (Fig. 1). It has been reported that maximal effect of translocation of NF-κB shows about 2–4 h and thereafter declines rapidly [19,20]. Thus, cells were cultured in the RWV bioreactor for 3 h and nuclear lysates were collected to assess NF-κB nuclear translocation and activation.

As shown in Fig. 2, the results from western blots revealed that the NF-κB p65 protein was significantly increased in the nuclear fraction under conditions of MG, as compared with DC. Mean nuclear translocation of p65 was 69% for MG and 46% for DC as compared with the TNF-α positive control ($p<0.05$). The increased nuclear translocation suggested by the Western blots were corroborated by increased DNA binding of p65 as shown by the ELISA results, which showed 66% for MG and 45% for DC (relative to TNF-α positive control) (Fig. 3). Thus, simulated MG leads to ~50% enhanced activation of NF-κB as compared with DC in a cardiac muscle cell line. It is probably based on our current results that enhanced activity of NF-κB under MG as compared with DC will lead to differential regulation of down-stream gene networks precipitating some of the physiological changes observed under conditions of microgravity. This claim is also supported by our recent study using DNA microarrays to show that genes involved in oxygen utilization and metabolic processes were significantly dysregulated under micro-g conditions as compared with unit-g conditions [6]. The present results let us conclude that simulated micro-g conditions regulated the translocation of a prevailing oxygen-sensitive transcription factor, NF-κB in a cardiomyocyte cell line. The physiological effect of NF-κB activation can be further investigated with focus on specific target genes.[2] For instance, c-myc, NADH quinine oxidoreductase, and glucose-6-phosphate 1-dehydrogenase were suggested to be MG regulated from our preliminary DNA microarray results [6] and are transcriptional target genes of NF-κB.

Since this study has been documented for heart muscle cells (H9c2), the current results should be extended to investigate specific physiological end points such as muscle atrophy. It has been reported that chronic activation of NF-κB is associated with various pathological conditions including muscle wasting [13]. In addition, it is evident that the regulation of gene expression is differentially responsive to exposure duration to MG [21]. Therefore, further characterization of time-dependent responses of NF-κB activation by MG may be needed to understand how the associated time-dependent changes in gene expression may play into chronic phenotypes such as muscle atrophy.

Future work is also needed to assess the effect of NF-κB activation under MG on levels of sarcomeric proteins (e.g., myosin) to link the genotypic changes into the possible physiological changes or effects of micro-g in mammalian cells and tissues [22]. In addition, several effects observed during space flights such as muscular weakness are also very similarly observed as a consequence of aging [23,24]. This lends potential to correlate the MG-evoked physiological changes with other diseases or syndromes.

Acknowledgments

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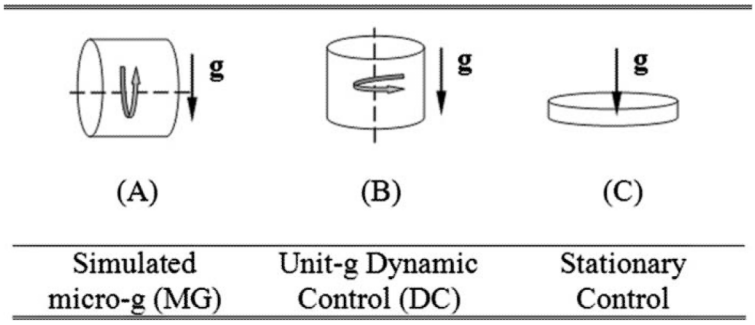

Fig. 1. Experimental design: (*a*) RWVs in the simulated MG and (*b*) the unit-g DC

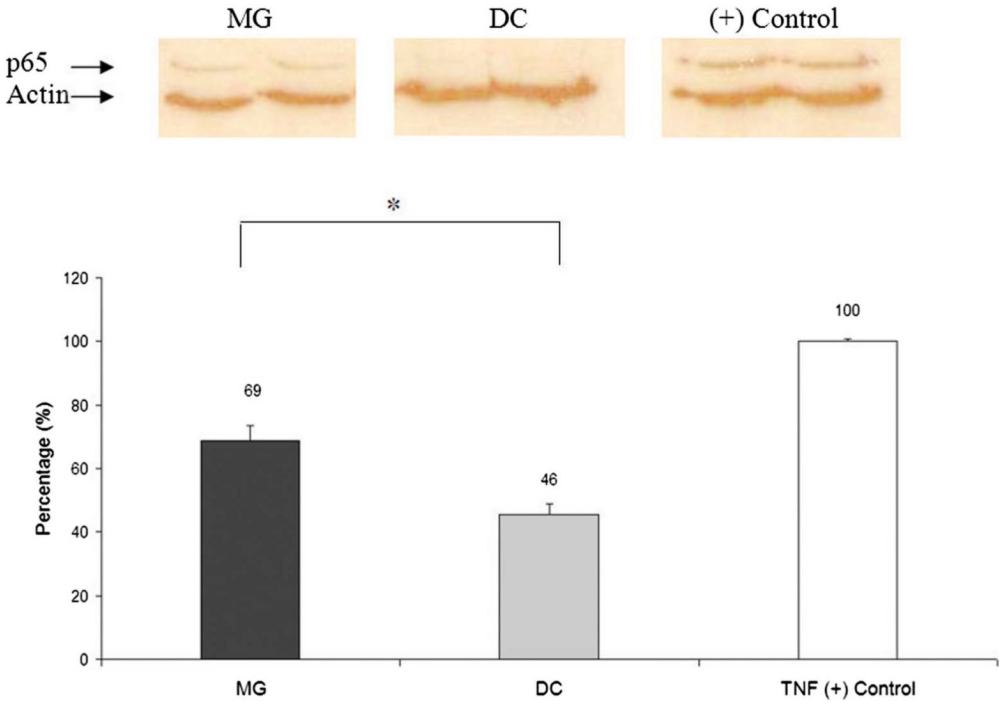

Fig. 2. Western blots result for NF-κB p65 in nuclear lysates of H9c2 cells (*p<0.05, data are expressed as mean ± SEM of three independent experiments)

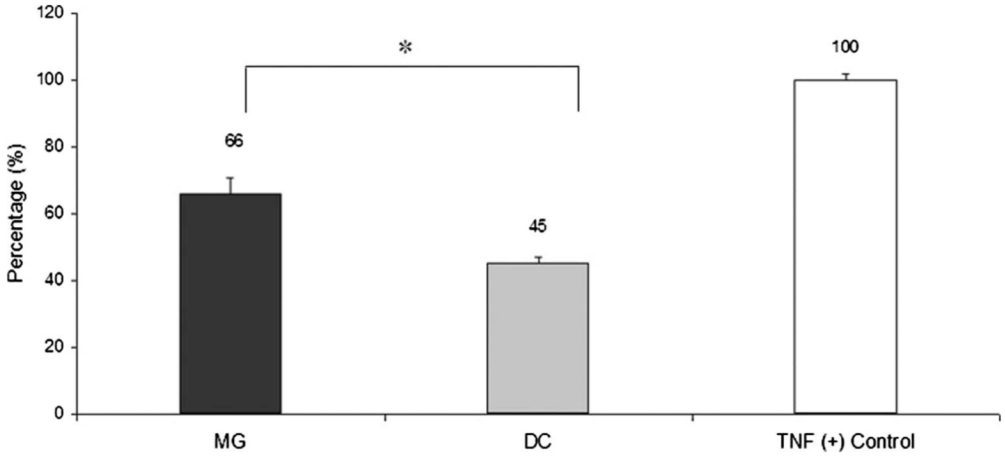

Fig. 3. ELISA result for NF-κB p65 in nuclear lysates of H9c2 cells (*p<0.05, data are expressed as mean ± SEM of three independent experiments)